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Bioremediation of Cadmium (Cd) in Batik Wastewater Using Different Carrier Media Containing Rhizobacteria

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Abstract: Batik wastewater is very dangerous for the environment and health because it contains heavy metals, such as Cadmium (Cd), derived from naphthol and indigosol dyes. Rhizobacteria have great potential to detoxify Cd on a laboratory scale. Therefore, they should be packaged in carrier media to ensure their long-term viability in the field. The carrier media used were peat, sawdust, and tofu solid waste. This research aimed to determine the most potential of three rhizobacteria as tolerant to Cd from 11 isolates, the best carrier media that can maintain rhizobacteria viability after freeze-dried, and the effectiveness of carrier media containing potential rhizobacteria in reducing Cd in batik wastewater. Furthermore, an experimental method with completely randomized and split-plot designs was used. Rb1, Rb3, and Rb6 were the most Cd-tolerant of the three rhizobacteria isolate tested. For the viability of each isolate, tofu solid waste and peat were the best carrier media at Rb, while sawdust and tofu solid waste were the best at Rb3 and peat at Rb6. Isolate Rb3C3 had the highest percentage value of degrading Cd at 85.1%, while others had less than 80%. Isolate rhizobacteria with a carrier media makes it easy to apply on a field scale, because it already contains a source of nutrients for bacterial growth and the packaging a longer shelf life.

Keywords: *Bioremediation, Cadmium, Carrier Media, Rhizobacteria*

INTRODUCTION

Batik is a piece of cloth made by drawing a design in the form of dots and lines using hot wax. In October 2009, UNESCO designated Indonesian Batik as a *Masterpiece of the Oral and Intangible Heritage of Man*, which has led to increased batik production. However, the wastewater in batik discarded without treatment can cause pollution (Syahputra & Soesanti, 2016). Cadmium (Cd) found in wastewater is a heavy metal that belongs to group IIB in the periodic system.

The metal of Cd in the human body will bind to metallothionein, and the interaction can inhibit the activity of the endothelial enzyme Nitric Oxide Synthase (eNOS). This enzyme plays a role in producing Nitric Oxide (NO), which causes a vasodilatation effect on blood vessels (Wijayanti & Lestari, 2017). According to Tamara (2013), cadmium in plants plays a role in metal homeostasis and detoxification.
The Cd in batik wastewater can be degraded through bioremediation, an alternative to reducing heavy metals or pollutants in the environment by using living organisms. One of these living organisms is rhizobacteria (Kour *et.al*., 2021). These bacteria have used a collection of Dr. Sri Lestari, S.Si., M.Si. with 11 isolates in the Microbiology Laboratory, Faculty of Biology, Jenderal Soedirman University.
Rhizobacteria require carrier media to be packaged, durable, and able to sustain their vitality (Widari, 2018). This research used peat, sawdust, and solid tofu waste as carrier media because it is easy to find, abundant, and cheap. Peat is the most widely used, and it is formed from an organic litter that decomposes anaerobically. However, the rate of adding organic matter is higher than decomposition (Sales da Silva *et al.,* 2020). Alternative carriers other than peat are sawdust and tofu solid waste. Media preservation can be conducted in two ways, including freezing and drying. The freezing consists of an ice coating covering the frozen commodities. However, this type of preservation has difficulties in terms of packaging. Drying removes water from the material under controlled conditions to produce dry products with minimal damage. A method suitable for microbial drying is freeze-drying for producing high-quality products (Widari, 2018).

Batik wastewater in Wangan River, Banyumas Regency, Central Java, contains heavy metals that exceed quality, namely Cd and chromium (Cr). The Cd and Cr in Wangan River of 0.018 mg/L and 0.231 mg/L have TSS, BOD, and COD levels of 540.13 mg/L, 540.42 mg/L, and 672.78 mg/L. In addition, batik wastewater contains 0.344 ppm rhodamine breaching dye, 0.179 ppm methylene blue, and 0.779 ppm methyl orange. This waste is produced from the Sokaraja Batik Center. The resulting naphthol and indigosol dyes contain large amounts of heavy metals such as zinc (Zn), Cr, and copper (Cu) (Lestari *et al*., 2017).

According to Lata *et al*. (2019), Pseudomonas can tolerate Cd up to 50 mg/L. A study on the common species to degrade Cd showed that *Pseudomonas putida* can degrade Cd by more than 80% in less than 5 minutes at pH 5.0-7.5. However, a study on carrier media containing rhizobacteria to degrade Cd in Batik wastewater has not been conducted. Therefore, this study aims to obtain the right carrier media for rhizobacteria in degrading Cd in batik wastewater. The objectives are to determine the most potential of three rhizobacteria isolates as tolerant to Cd from 11 isolates, the best carrier media that can maintain rhizobacteria viability after freeze-dried, and the best carrier media for the effectiveness of carrier media containing potential rhizobacteria in reducing Cd. This research provived benefit of isolate rhizobacteria with a carrier media makes it easy to apply on a field scale. Carrier media for rhizobacteria already contains a source of nutrients for bacterial growth and the packaging a longer shelf life.

METHODS

Research Method

The selection of 11 rhizobacteria isolates was carried out by a descriptive survey method using a tolerance test on Cd. The ability to degrade Cd in batik wastewater was conducted experimentally using a completely randomized design (CRD) with a split-plot design. The independent variables were rhizobacteria isolates and carrier media. In contrast, the dependent variable was the ability of rhizobacteria isolates to degrade Cd. The main parameters observed were the tolerant value on cadmium, the viability value of potential rhizobacteria in carrier media, and the Cd concentration value, while the supporting parameter was pH.

Batik Wastewater Collection

Batik wastewater was taken from the batik industry center in Sokaraja Batik Center, Sokaraja District, Banyumas Regency, Central Java Province. About 5 liters of the wastewater was taken using a jerrycan.

Rhizobacteria Isolate Retrieval

Collection of rhizobacteria were taken from the Laboratory of Microbiology, Faculty of Biology, Jenderal Soedirman University totaling 11 isolates. They are Rb1, Rb2, Rb3, Rb4, Rb5, Rb6, Rb7, Rb8, Rb10, Rb11, and Rb12.

Sterilization of tools and materials

Tools and materials were sterilized in an autoclave at a temperature of 121°C and a pressure of 2 atm for 15 minutes.

Purification and Reculture of Rhizobacteria Isolates

Rhizobacteria isolates were purified using the quadrant streak method on a petri dish containing NA medium and were incubated for 2x24 hours at room temperature. Subsequently, rhizobacteria isolates

were recultured on an NA slant medium and incubated for 1x24 hours. Finally, they were grown in 9 mL of NB medium and incubated for 1x24 hours to obtain inoculum.

Activation of Inoculum

The culture in 9 mL NB was inoculated into an Erlenmeyer containing 100 mL NB. The activation process was carried out with an incubation time of 1x24 hours. This active culture will be the inoculum.

Tolerance Test of Rhizobacteria Isolates on Cadmium

Aseptically, 0.1 mL of 11 inoculums were inoculated into 5 mL of NB media with a $CdCl_2$ content of 0.1 mg/L for 24 hours. The culture results were measured using a UV-Vis spectrophotometer at 600 nm. Furthermore, 3 cultures were selected with a small final value (control-treatment), which indicated that there was bacterial growth tolerant of cadmium.

Preparation Rhizobacteria Growth Curve using Total Plate Count Method

Rhizobacteria growth curves were made for isolates with a high tolerance for cadmium. The Cadmium tolerant isolate culture used was active. A total of 1 mL of inoculum was inoculated into 9 mL of sterile distilled water and then homogenized. Subsequently, the dilution was carried out up to 10^{-6}. A total of 1 ml of the results were taken and then inoculated into NA media using a pour plate and then incubated for 1x24 hours at room temperature. Observations on the TPC value were conducted every 2 hours from 0 to 24 hours. After incubation, the number of bacterial colonies and populations was calculated using the TPC formula:

$$\Sigma\left(\frac{\text{cfu}}{\text{mL}}\right) = \text{colony average x } \frac{1}{\text{dilution factor}} \, x \, \frac{1}{\text{pour plate}}$$

Preparation of Carrier Media

The carrier media of peat, sawdust, and solid tofu waste were roasted, blended, and filtered with a size of 425μm before autoclaving at 121°C, 2 atm for 15 minutes. The carrier media was mixed with the potential isolates provided for every 15 mL of broth culture (10^8 cell/mL) on 50 g of the medium.

Drying Process

The carrier media produced contains an amount of water needed to be dried. A freeze dryer with an operating temperature of 37-39°C, a vacuum pressure of 16 cmHg up to 85% humidity, and a time of 2 hours was used.

Viability Test of Rhizobacteria

A viability test was conducted by incubating the carrier media containing rhizobacteria for 30 days at room temperature. Every 10 days intermittent, the total population was counted using the TPC method. About 1 g the carrier media containing rhizobacteria were put into 9 mL of sterile distilled water and then homogenized. Subsequently, serial dilutions were performed up to 10^{-7} dilutions. A total of 1 ml of the dilution was taken and inoculated on NA medium by pour plate method, then incubated for 1x24 hours. The number of bacterial colonies and populations were calculated using the TPC formula.

Bioremediation Test of Batik Wastewater by Carrier Media Contains Rhizobacteria at Lab Scale
Carrier media containing potential rhizobacteria isolates of as much as 10 grams were added to 100 mL of batik wastewater, then incubated for 14 days. After the incubation period, the concentration of metal Cadmium was then measured.

The incubated solution was separated between bacteria and wastewater using a 0.22 μm filter membrane or centrifuge at 4500 rpm for 15 minutes. About 50ml of the results were added to a 100 ml erlenmeyer flask. The solution was heated with a hot plate at 180°C until 10-20 mL remained. Furthermore, it was added with 5 mL of concentrated HNO_3 and 2 mL of 20% HCl then heated until the solution became clear. It was filtered with Whatman paper no. 42, and the filtrate obtained was then diluted with distilled water using a 50 mL volumetric flask until the mark was measured. The absorbance of CD was measured using AAS at a wavelength of 228.8 nm and lamp currents of 8 mA. Calculation of the percentage Cd degradation uses the formula:

$$\%\ \text{Cd Degradation} = \frac{C(a) - C(b)}{C(a)} \text{x } 100\%$$

Details :
C(a)= initial metal concentration (ppm)
C(b)= final metal concentration (ppm)

The initial concentration was the metal concentration before the biosorption treatment. The final concentration is the metal concentration after biosorption treatment.

Measurement of pH
Measurements of pH used pH universal to immerse the sample solution and compare the results with the existing determination.

Data analysis
Cadmium concentration value data were analyzed using analysis of variance (ANOVA) at a 95% confidence level. The significantly different results were continued with the Duncan Multiple Range Test (DMRT) to determine the optimum carrier media.

RESULT AND DISCUSSION

The tolerance test of rhizobacteria isolates on cadmium was at 0.1 mg/L $CdCl_2$. Rhizobacteria isolates Rb1, Rb2, Rb3, Rb4, Rb5, Rb6, Rb7, Rb8, Rb10, Rb11 and Rb12 were tolerant to Cd. This was indicated by the growth of rhizobacteria when given 0.1 mg/L $CdCl_2$. The isolates of rhizobacteria tolerance to cadmium are shown in Table 1. The isolates Rb1, Rb3, and Rb6 having high tolerant to Cd. The isolates Rb2, RB8 and Rb12 were tolerant to Pb while Rb6, Rb7 and Rb12 tolerant to Zn (Oedjijono *et al.,* 2022). According to Winardi *et.al.,* (2018), bacteria that have potential as bioremediation agents can survive in heavy metals environments. They require heavy metals as cofactors to produce enzymes useful for pollutant degradation. The rhizobacteria isolate used were Rb1, Rb3, and Rb6, which were indicated by the smallest final values compared to others. The final values were 0.1493, 0.1850, and 0.1843, while the detailed description can be seen in Table 1.

Table 1. Result of Rhizobacteria Isolates Tolerance Test on Cd

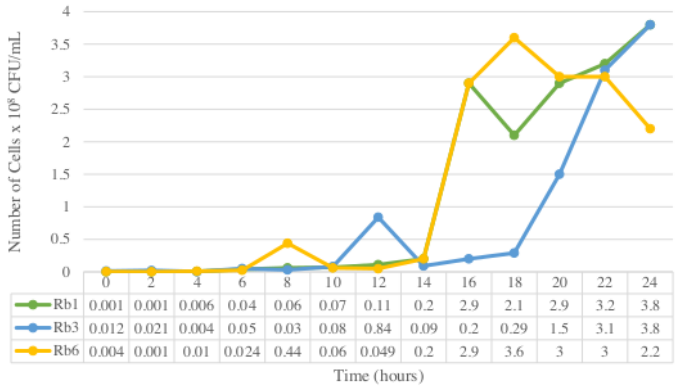
Rhizobacteria Isolates	Population Density Absorbance Based on OD		Increased Rhizobacteria Growth (Control-Treatment)
	Control	Treatment	

Rb1	0.152	0.0027	0.1493
Rb2	0.211	0.0027	0.2083
Rb3	0.188	0.0030	0.1850
Rb4	0.192	0.0047	0.1873
Rb5	0.192	0.0027	0.1893
Rb6	0.186	0.0017	0.1843
Rb7	0.196	0.0057	0.1903
Rb8	0.237	0.0083	0.2287
Rb10	0.285	0.0170	0.2680
Rb11	0.246	0.0050	0.2410
Rb12	0.828	0.4667	0.3613

The population density of rhizobacteria needs to be searched to determine when rhizobacteria can be used optimally to reduce Cd. According to Wongchawalit *et al.* (2020), the optimal number of rhizobacteria population densities in heavy metal bioremediation is 10^8 CFU/m. Setiawati (2014) stated that the determination of the growth curve serves to see the phase of rhizobacteria. The bacterial growth curve is divided into lag (slow phase), exponential growth (fast-growth phase), stationary (static phase), and decline phases (population decline phase).

The number of rhizobacteria cells varied based on the incubation time and isolate type. For example, at the incubation time of 12 hours, the growth of isolates Rb1, Rb3, and Rb6 showed a lag phase with the number of cells $0.11x10^8$ CFU/mL, $0.84x10^8$ CFU/mL, and $0.049x10^8$ CFU/mL (Figure 1.). The lag phase is the time rhizobacteria need to adapt to their new environment. In Rb1 and Rb6 isolates, the exponential phase occurred at an incubation time of 14-16 hours of growth, while Rb3 isolates took 18-22 hours of growth. The exponential phase covers cell division, where the cell will divide until the maximum number is reached.

The stationary phase in Rb1, Rb3, and Rb6 isolates occurred at an incubation time of 16-24, 22-24, and 16-22 hours of growth (Figure 1.). The stationary phase describes the accumulation of metabolites resulting from cell metabolic activity, and nutrient content begins to run out. At the incubation time of 22-24 hours, the growth of Rb6 isolates showed a decline phase. In this phase, more dead cells are formed than new cells (Sharah *et al.*, 2015). Therefore, isolates of Rb1, Rb3, and Rb6 will be optimally used in heavy metal bioremediation when incubated until they reach the stationary phase.



	0	2	4	6	8	10	12	14	16	18	20	22	24
Rb1	0.001	0.001	0.006	0.04	0.06	0.07	0.11	0.2	2.9	2.1	2.9	3.2	3.8
Rb3	0.012	0.021	0.004	0.05	0.03	0.08	0.84	0.09	0.2	0.29	1.5	3.1	3.8
Rb6	0.004	0.001	0.01	0.024	0.44	0.06	0.049	0.2	2.9	3.6	3	3	2.2

Figure 1. Growth Curve of Rhizobacteria Isolates Rb1, Rb3 and Rb6 based on TPC on NB Media

The viability of Rb1, Rb3, and Rb6 isolates in various carrier media showed a density value of 10^8 CFU/mL on day 30 as indicated in Figure 2. The growth and survival of bacterial isolates can also be influenced by indigenous microorganisms contained in the carrier media. Therefore, it is necessary to sterilize the carrier media to prevent competence between bacterial isolates and indigenous microorganisms. According to Yanti *et al*. (2017), a carrier media with a smaller particle size can increase its surface area to protect bacteria from drying out.

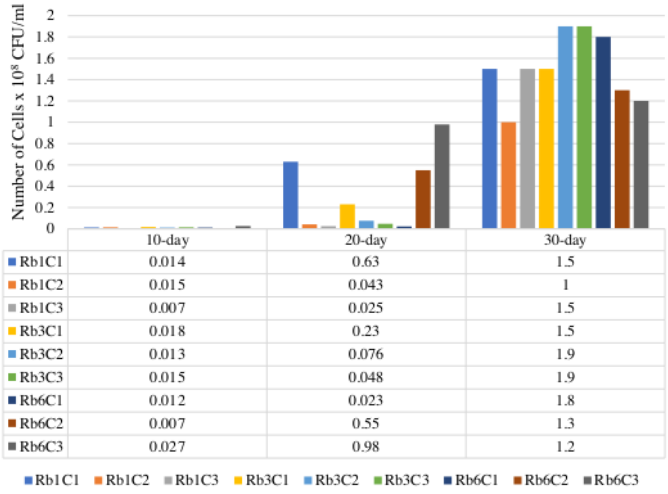
Determination viability of Rb1, Rb3, and Rb6 isolates on peat, sawdust, and tofu solid waste carrier media based on the number of colonies during a 30-day storage period were conducted using the TPC method (Figure 2.). Several cells in a population may die during biomass production and initial storage. This was indicated by a decrease in the number of bacterial isolates Rb1, Rb3, and Rb6 at the beginning and after being applied to the carrier medium on the 10-day. Isolates Rb1, Rb3 and Rb6 had initial colony numbers from 2.9-3.1x10^8 CFU/mL (Figure 1.) to 0.7-2.7x10^6 CFU/mL (Figure 3.). According to Sales da Silva *et al.,* (2020), bacteria undergo an adaptation phase of life in a solid carrier medium.

Figure 2. Rhizobacteria viability test on carier media

Rb1 isolate carrying peat and tofu solid waste had the highest value at $1.5x10^8$ CFU/mL, while that of Rb3 isolate carrying sawdust and tofu solid waste was $1.9x10^8$ CFU/ mL. The isolate Rb6 peat carrier media had the highest value of $1.8x10^8$ CFU/mL. Malusa *et al*. (2012) reported that peat had been commonly used as a carrier medium for rhizobacteria due to its abundant availability and history of field trials. Sawdust proved useful for inocula production containing different bacterial strains (Lennox *et al*., 2019). Solid waste from the tofu industry contains highly organic substances. One of them can be used as a carrier medium for bacteria (Faisal *et al*., 2016).

Based on Figure 3. it can be seen that each treatment experienced an increase in the number of different bacteria during the 30-day storage period. The number of cells in the Rb1C1 to Rb6C3 treatment significantly increased during each storage period. A storage period of 30 had the highest number of bacterial cells. Rb1C1, Rb1C2, Rb1C3, Rb3C1, Rb3C2, Rb3C3, Rb6C1, Rb6C2, Rb6C3 has the highest cell count of $1.5x10^8$ CFU/mL, $1.0x10^8$ CFU/mL, $1.5x10^8$ CFU/mL, $1.5x10^8$ CFU/mL, $1.9x10^8$ CFU/mL, $1.9x10^8$ CFU/mL, $1.8x10^8$ CFU/mL, $1.3x10^8$ CFU/mL and $1.2x10^8$ CFU/mL (Figure 3.) . According to Sales da Silva *et al*., (2020), changes in the number of bacteria populations are influenced by nutrition, temperature, pH, aeration (oxygen availability), and toxic compounds, such as oxalic acid, aliphatic hydrogen, and other hazardous chemicals.

Wongchawalit *et al*. (2020) stated that the optimal number of rhizobacteria population densities in heavy metal bioremediation is 10^8 CFU/mL. Therefore, the isolates that have been inoculated into various carrier media will be optimal when used for batik wastewater after incubation for 30 days to reduce the Cd content.



	10-day	20-day	30-day
Rb1C1	0.014	0.63	1.5
Rb1C2	0.015	0.043	1
Rb1C3	0.007	0.025	1.5
Rb3C1	0.018	0.23	1.5
Rb3C2	0.013	0.076	1.9
Rb3C3	0.015	0.048	1.9
Rb6C1	0.012	0.023	1.8
Rb6C2	0.007	0.55	1.3
Rb6C3	0.027	0.98	1.2

Figure 3. Population Density of Rb1, Rb3 and Rb3 Isolates in Different Carrier media and stored within 30 days

Isolate Rb1, Rb3 and Rb6 can reduce Cd in batik waste. The Rb3C3 had a percentage Cd degradation value of 85.1%, while others only had less than 80% (Figure 4.). According to Hardiani *et al*. (2011), factors that affect the percentage decrease in Cd concentration are microbial activity, nutrition, acidity, and environmental factors. Winardi *et al*. 2020 added mercury bioremediation in mining areas

using soil bacteria influenced by pH, aeration and nutrients. Resistance of Cd in rhizobacteria is primarily based on the active efflux of metal ions by P-type ATPases, cation diffusion facilitator (CDF) transporters, CBA transporters, and chemiosmosis. P-type ATPases transport proteins against the concentration gradient using energy provided by ATP hydrolysis. Furthermore, CDF is a group of transporters that can catalyze heavy metals' influx or efflux. P-type ATPases and CDF transporters are commonly found among different bacterial species. In contrast, the presence of a CBA transporter is exceptional and indicates a high level of resistance to heavy metal ions (Sharma & Archana, 2016).

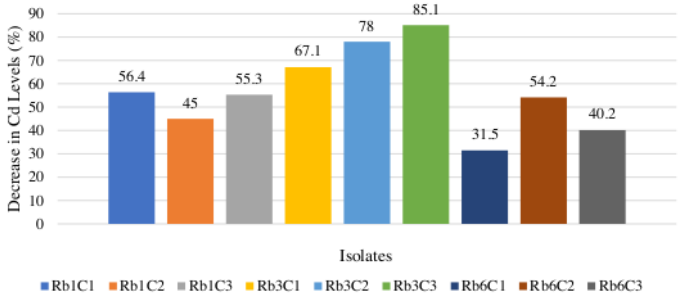


Figure 4. Result of Percentage Cd Degradation in Batik Wastewater (C1: peat, C2: sawdust: C3: tofu solid waste)

Analysis of variance was needed to determine the mean difference between groups or treatments, and the final result is the calculated F value. There is a considerable difference in the mean for all groups when the F-calculated value exceeds the F-table (Setiawan, 2019). Based on Analysis of Variance the rhizobacteria isolate, carrier media, and the interaction between rhizobacteria isolate and carrier media had a significant effect at the 95% confidence level. This was indicated by the F-calculate value that was greater than the F-table. The rhizobacteria isolate, carrier media, and the interaction between rhizobacteria isolate and carrier media greatly influence the Cd bioremediation process in batik wastewater.

The Duncan Multiple Range test was needed to determine which carrier media has more influence on the bioremediation process. Based on Table 3. peat was the most influential carrier medium in the bioremediation process. Shvartseva *et al*. (2022) also reported that peat potensial to remove copper from aqueous. Additionally, the polar nature makes it ideal for the adsorption of dissolved substances, including metals and organic compounds. This character allows peat to purify wastewater contaminated with dissolved metals.

Table 3. Result of duncan multiple range test on Different Carrier Media

Carrier Media	Average
Peat	0.1879 b
Sawdust	0.1590 a
Tofu solid waste	0.1549 a

Table 4.Value of pH in Each Treatment

Treatment	pH value
Batik Wastewater	13.0
Rb1 in Peat	9.0
Rb1 in Sawdust	9.0
Rb1 in Tofu solid waste	9.0
Rb3 in Peat	9.0

Rb3 in Sawdust	9.3
Rb3 in Tofu solid waste	8.0
Rb6 in Peat	9.3
Rb6 in Sawdust	9.0
Rb6 in Tofu solid waste	8.0

According to *Perda Jateng No. 5 Tahun 2012*, the pH value following the batik wastewater quality standard is 6-9. The pH values for each treatment can be seen in Table 4. with a pH value of batik wastewater of 13. The treatment of Rb1 in Peat, Rb1 in Sawdust, Rb1 in Tofu solid waste, Rb3 in Peat, Rb3 in Tofu solid waste, Rb6 in Sawdust, and Rb6 in Tofu solid waste follows the quality standard of batik wastewater. Metal ions will spontaneously react with hydroxides to form metal-hydroxide bonds at alkaline pH. In contrast, there will be competition between metals and H^+ ions at an acidic pH to bind to microbial cell walls. This causes metal accumulation in microbial cells at neutral pH greater than an acidic or alkaline. Therefore, extremely acidic or alkaline pH can kill bacteria since they thrive in neutral environments (Maulana *et al*., 2017).

CONCLUSION

Rhizobacteria isolate of Rb1, Rb2, and Rb6 are tolerant to Cd. The best carrier media for Rb1 were peat and tofu solid, Rb3 were sawdust and tofu solid, and Rb6 was peat. Furthermore, isolate Rb3 with tofu solid had the highest percentage value of degraded Cd at 85.1%. Rhizobacteria with carrier media facilitates field application and has a long shelf life.

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